Supporting Information

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SI Methods

Culture and Transplantation of hESCs. H1, H9, and H9^{DF} hESCs were initially maintained on top of murine embryonic fibroblasts feeder (MEF) layers seeded onto 0.1% gelatin-coated plastic dishes and inactivated by γ-irradiation (6,000 RAD). hESCs were maintained in hESC medium containing 80% Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Invitrogen), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 0.1 mM non-essential amino acids (Invitrogen), 20% Knockout Serum Replacement (Invitrogen), and 8 ng/ml human basic fibroblast growth factor (bFGF; Invitrogen) (1). MEF were derived from CF-1 E12.5 embryos as previously described (2). The hESC culture medium was changed daily and hESCs were passaged every 4–5 days. hESC differentiation was induced by embryoid body (EB) formation. hESC colonies were dispersed into cell aggregates using 1 mg/ml collagenase IV. These aggregates were then cultured in suspension in ultra-low attachment plates (Corning) in hESC differentiation medium, consisting of DMEM high glucose supplemented with 20% FBS (HyClone), 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 0.1 mM non-essential amino acids for 7 days. Then, EBs were transferred to 10-cm culture dishes coated with 0.1% gelatin and cultured for an additional 7 days. hESC differentiation medium was changed every two days.

FACS Analysis of hESC Surface Marker Expression. H1, H9, and H9^{DF} hESCs were trypsinated, washed, and incubated with PE-conjugated mouse anti-human HLA-ABC (G46–2.6), β_2-microglobulin (Tü99), HLA-DR, DP, DQ (Tü39), or their respective isotype control antibodies (all BD Biosciences) in FACS buffer (PBS 2% FCS) for 45 min at 4°C. Cells were washed, incubated with 7-amino-actinomycin D (7-AAD) cell viability solution (eBiosciences), and analyzed on a FACSCalibur system (BD Biosciences). For analysis of pluripotency markers, a similar protocol was followed, using PE-conjugated anti-human SSEA-1 (MC-480) and purified anti-human SSEA-4 (MC-813–70) antibodies (R&D Systems). The latter followed by incubation with PE-conjugated anti-IgG secondary antibody (eBioscience) for 30 min at 4°C.

Quantification of IFN-γ Secretion by hESC. Cytokine Antibody Array (Raybiotech) were used to identify the H9 and H9^{DF} hESC secretion profiles of IFN-γ. Membranes were covered with 24-hour supernatant of H9 hESC, H9^{DF} hESC, medium alone as well as medium containing 25 ng/ml recombinant IFN-γ (Peprotech) as a positive control. Membranes were developed according to the manufacturer's protocol. Integrated densities were calculated using National Institutes of Health imageJ 1.38. Values were normalized to the integrated positive control on each membrane.

Animal Experiments. Six- to 10-week-old female BALB/c (wild-type), C57BL/6J-*Tyr*$^{c-2J}$/J (C57BL/6 albino or C67Bl/6a), NOD.CB17-*Prkdc*^{scid}/J (NOD/SCID), B6.129S2-*Cd4*^{tm1Mak}/J (CD4 knockout), B6.129S2-*Cd8a*^{tm1Mak}/J (CD8 knockout) mice (The Jackson Laboratory), and BALB/c Nude (T cell deficient, Charles River laboratories) mice were housed at no more than five per cage in our American Association for Accreditation of Laboratory Animal Care-approved facility with 12:12-h light-dark cycles and free access to standard rodent chow and water. All animal procedures were approved by the Animal Care and Use Committee of Stanford University.

Tissue Collection, Immunofluorenscent and Histological Analysis. Explanted muscles were fixed in 2% paraformaldehyde for 2 h at room temperature and cryoprotected in 30% sucrose overnight at 4°C. Tissue was frozen in optimum cutting temperature compound (OCT compound, Sakura Finetek) and sectioned at 5 μm on a cryostat. Serial sections were blocked and incubated with hamster anti-CD3 (clone G4.18) (BD Biosciences) for 1 h at room temperature, followed by goat anti-hamster Texas Red (Santa Cruz Biotechnology) Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) and analyzed with a Leica DMRB fluorescent microscope (Leica Microsystems, Frankfurt, Germany). Hematoxylin and Eosin staining (Sigma) was performed according to established protocols. For histopathogical evaluation of hESC survival 6 sections of each explanted muscle were stained with H&E and carefully analyzed by a blinded pathologist (H.V.).

Quantification of Graft Infiltrating Cells. Gastrocnemius muscles were surgically explanted, minced, and digested for 2 h in Collagenase D (2 mg/ml; Worthington Biochemical) at room temperature in RPMI 1640 media (Sigma Chemical Co.) with 10% FCS (FCS; Life Technologies). Muscle cell suspensions were ran through a 70-μm cell strainer, washed in FACS buffer (PBS 2% FCS), and incubated with PE-conjugated CD3e (145–2C11), CD8a (53–6.7), Mac-1/CD11b (M1/70), and allophycocyanin (APC)-conjugated CD4 (GK1.5), B220 (RA3–6B2), and Gr-1 (RB6–8C5) antibodies (CD4 and CD8: eBiosciences, all others: BD Bioscience) for 45 min at 4°C. Cells were washed, incubated with 7-AAD cell viability solution (eBiosciences), and analyzed on a FACSCalibur system (BD Biosciences).

Quantification of Humoral Immune Response. Sera from recipient mice were decomplemented by heating to 56°C for 30 min and subsequently diluted by 33% in PBS containing 3% FCS and 0.1% NaN_3. Equal amounts of sera and hESC (1×10^6 cells per milliliter) suspensions were incubated for 30 min at 4°C and washed with PBS through a calf-serum cushion. IgM xenoreactive antibodies were stained by incubation of the cells with PE-conjugated goat antibodies specific for the Fc portion of mouse IgM (BD Bioscience) at 4°C for 45 min. Cells from all groups were washed twice with PBS containing 2% FCS and then analyzed on a FACSCalibur system (BD Biosciences). Fluorescence data were collected by use of logarithmic amplification and expressed as mean fluorescent intensity.

Immunosuppressive Therapy Protocol. Adult female BALB/c mice ($n = 30$) were randomized to receive Tacrolimus (TAC; Sigma-Aldrich), sirolimus (SIR; Rapamune oral solution; Sigma-Aldrich), and mycophenolat mofetil (MMF; Roche). All drugs were administered once daily by oral gavage, using the following doses for TAC, SIR, and MMF: 4, 3, and 30 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively, to achieve drug serum levels comparable to clinical trough levels of 10–15 ng/ml for TAC and SIR and 3.5–5.5 ng/ml for MMF. Blood was drawn during animal sacrifice and 12- or 24-hour drug trough levels were quantified by high-performance liquid chromatography (HPLC) as described earlier (3).

1. Amit M, *et al.* (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* 227:271–278.
2. Thomson JA, *et al.* (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
3. Taylor PJ, Salm P, Lynch SV, Pillans PI (2000) Simultaneous quantification of tacrolimus and sirolimus, in human blood, by high-performance liquid chromatography-tandem mass spectrometry. *Ther Drug Monit* 22:608–612.

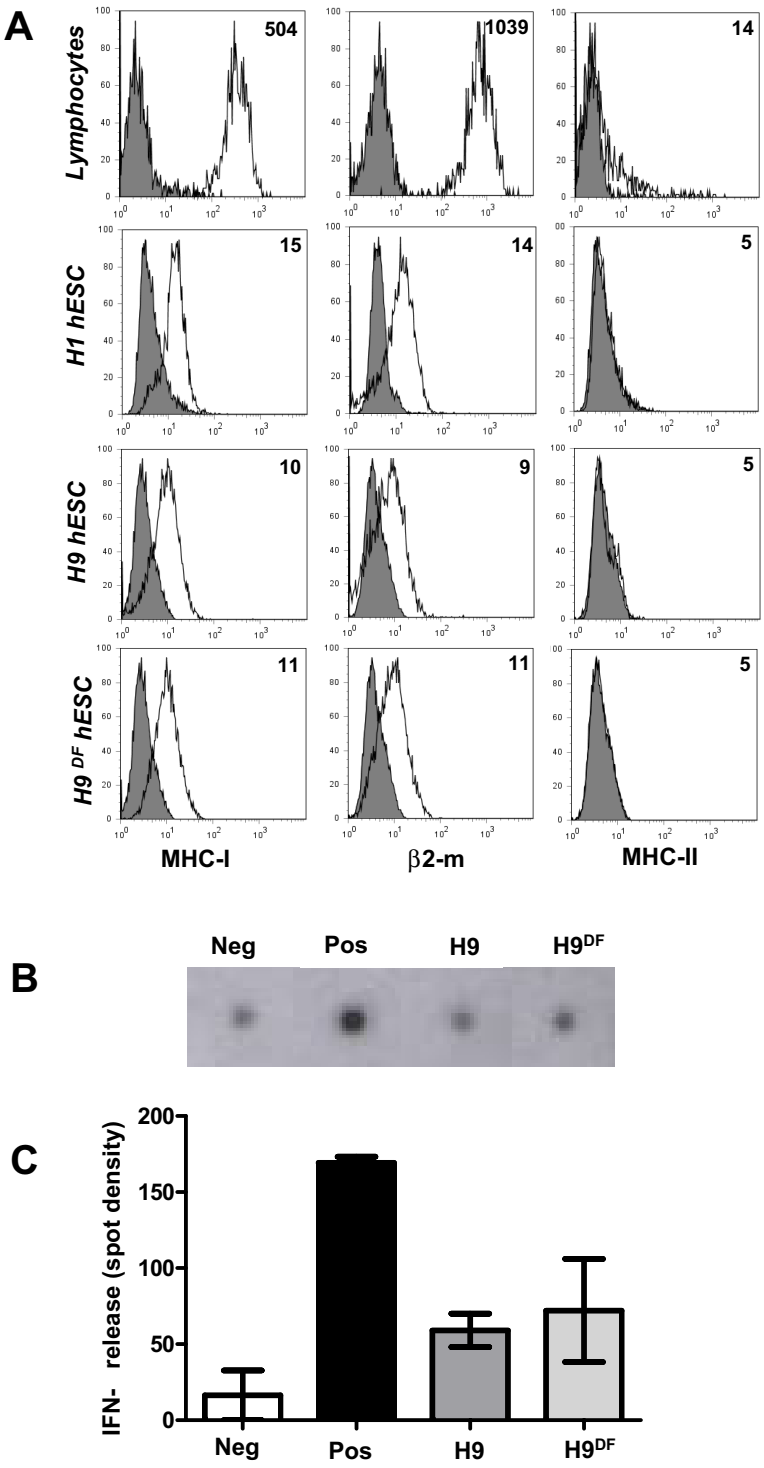

Fig. S1. Immunological characterization of H9^{DF} hESCs compared to non-transduced controls. (*A*) Compared with human lymphocytes as a positive control, both H1 and H9 hESCs as well as transduced H9^{DF} hESCs express low amounts of MHC-I and β2-microglobulin, and remain negative for MHC-II. Mean fluorescent intensity (MFI) is shown in the upper right corner of each panel. Results are representative of three independent experiments. (*B*) Representative images and (*C*) graphical representation of the cytokine antibody array show no difference in IFN-γ secretion by H9 or H9^{DF} hESCs (neg = negative control, hESC medium; pos = positive control, medium containing recombinant human IFN-γ at 25 ng/ml).

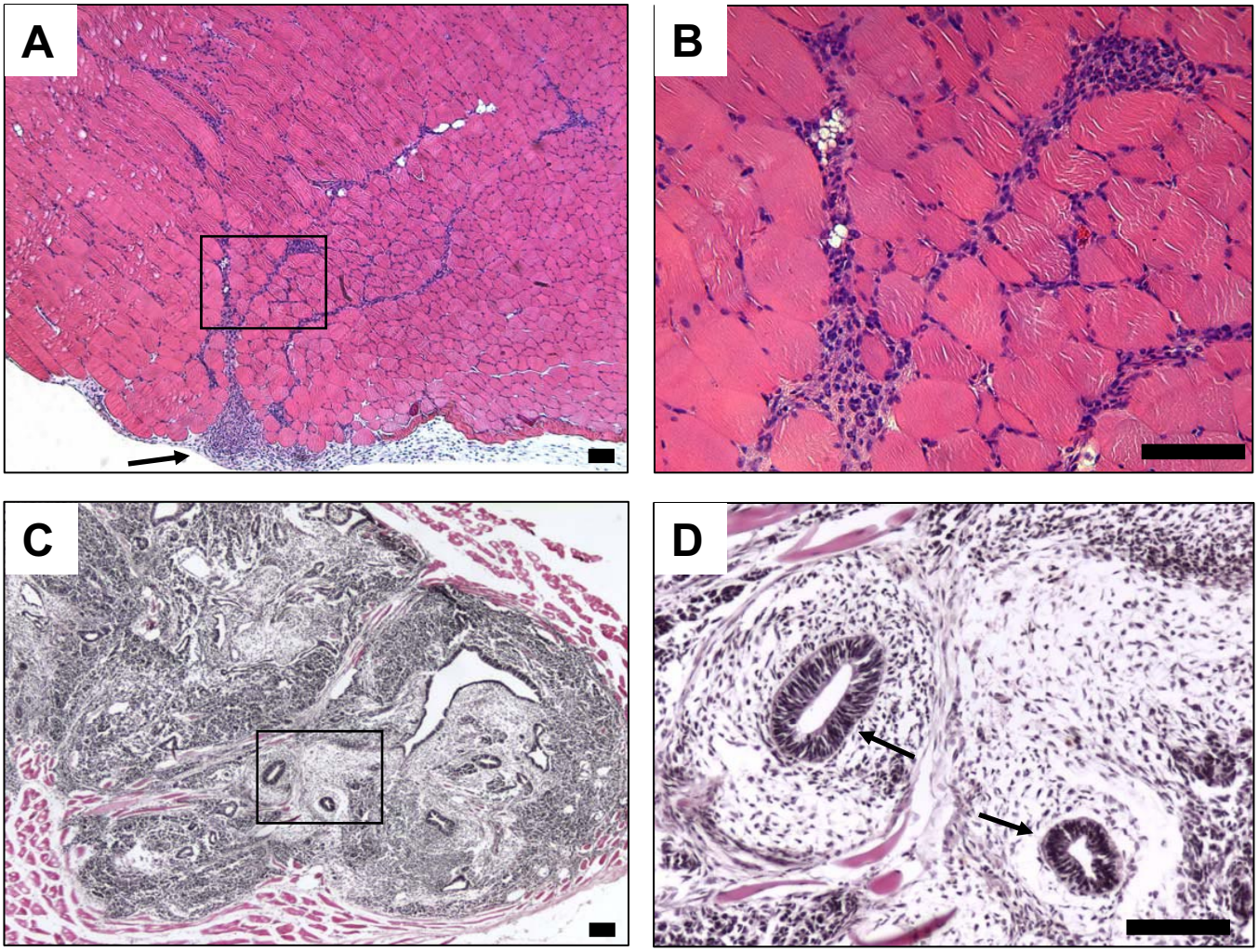

Fig. S2. Histopathological evaluation of hESC survival. (*A*) Tissue sections of BALB/c recipient muscles show endomysial mixed inflammatory cell infiltrates, sometimes involving the perimysium and adjacent soft tissue, representing the injection track (black arrow). (*B*) On higher magnication, infiltrates consisting of mixed mononuclear and granulocyte infiltrates can be observed. No hESCs or cells with morphology that would suggest anything other than inflammation could be detected. (*C*) Explanted muscles of NOD/SCID animals at 42 days after transplantation demonstrates formation of intramuscular hESC-derived teratomas, composed out of tissue representing the three germ layers. (*D*) On higher magnification, endodermal derived glandular epithelium (black arrows) surrounded by mesodermal derived mesenchyme can be detected. (Scale bars, 50 μm.)

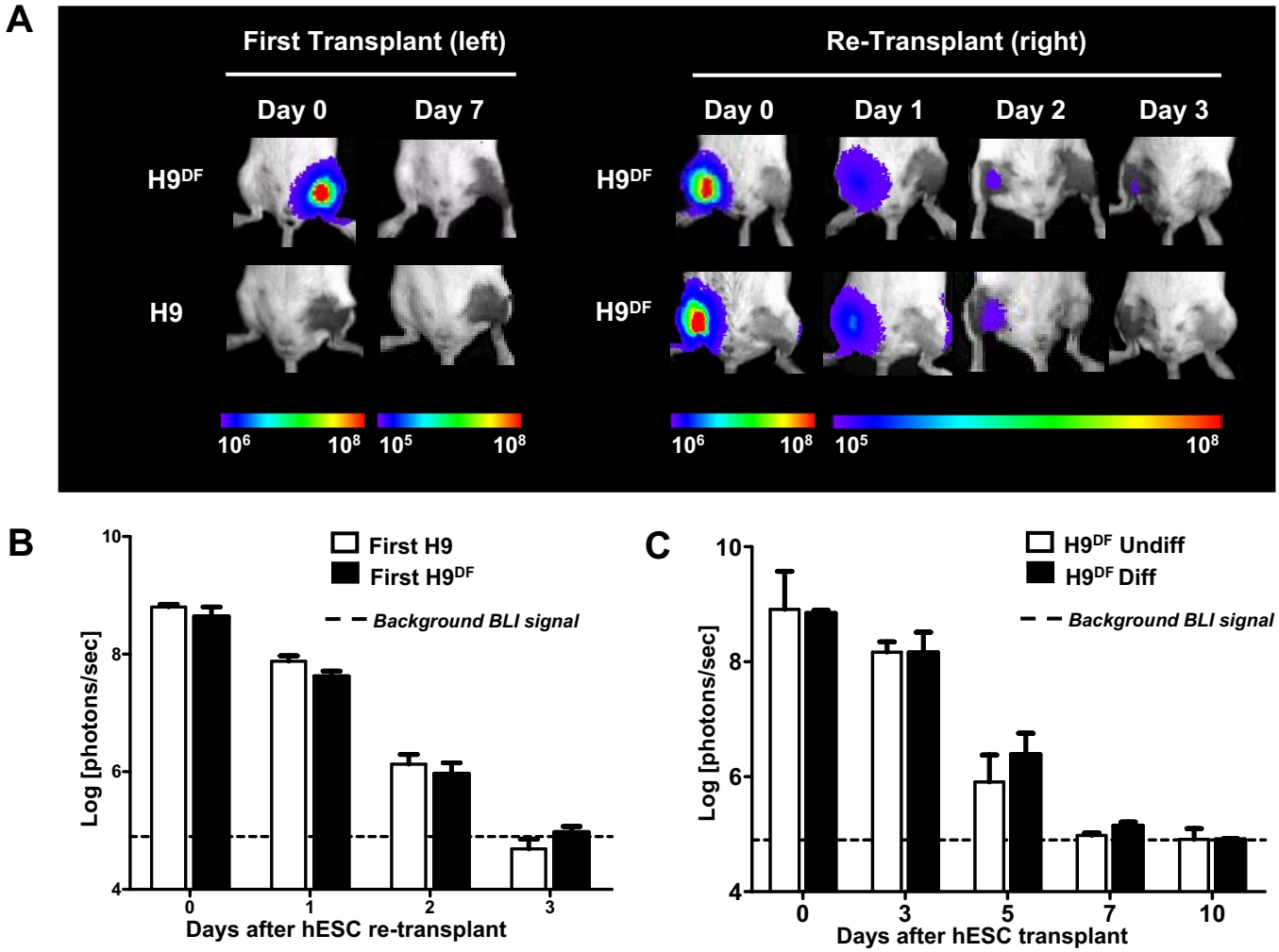

Fig. S3. Similar hESC death after re-transplantation following primary stimulation with either transduced hESCs or non-transduced hESCs. (*A*) Representative BLI images (color scale bar values are in photons per second per squared centimeter per steradian (sr)) and (*B*) graphical representation shows a similar trend in BLI signal loss in the 3 days following secondary transplantation of $H9^{DF}$ hESCs in BALB/c animals, after primary stimulation with either non-transduced H9 hESCs ($n = 3$) or transduced hESCs ($n = 6$) two weeks earlier. (*C*) Graphical representation of BLI signals comparing survival of undifferentiated $H9^{DF}$ hESC ($H9^{DF}$ undiff) vs. 14-day differentiated $H9^{DF}$ hESC ($H9^{DF}$ diff) following transplantation. No significant difference in cell survival was found.

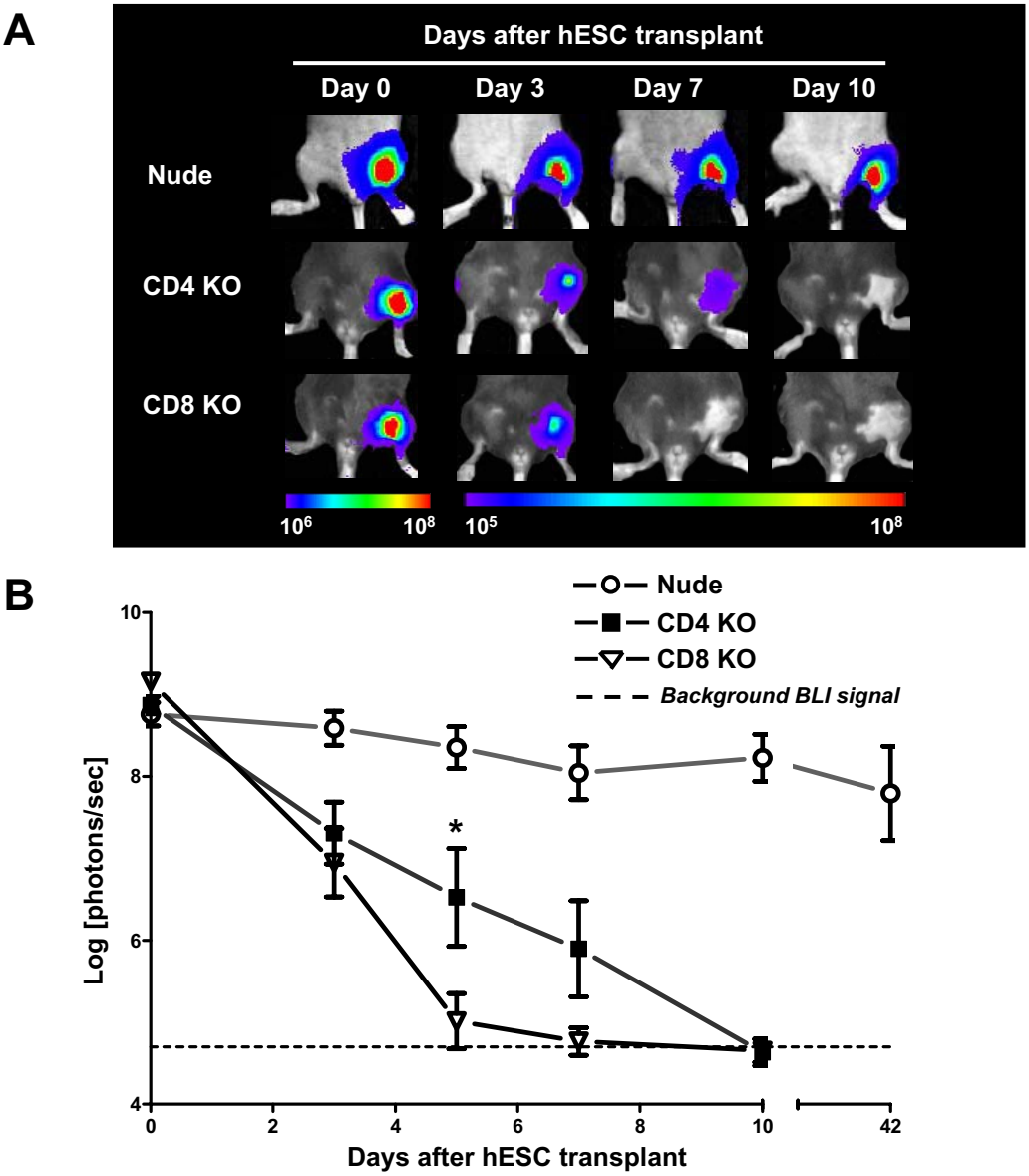

Fig. S4. Role of T cell subsets in mouse anti-hESC immune rejection. (*A*) Representative BLI images of H9^{DF} hESCs transplanted into different immunodeficient mouse strains show survival of the donor cells in Nude mice up to 42 days following transplantation, suggesting an important role for T cells in mouse anti-hESC rejection. Although hESCs are eventually rejected in both CD4 knockout (CD4-KO) and CD8 knockout (CD8-KO) mice, there is significantly longer survival of hESC in CD4-KO animals. Color scale bar values are in photons per second per squared centimeter per sr. (*B*) Graphical representation of BLI of hESC survival in the three groups ($n = 4$ or 5 per group). *, $P < 0.05$

Table S1. Immunosuppressive treatment dosages and serum drug trough levels

Drug	Dosage, $mg \cdot kg^{-1} \cdot d^{-1}$	Trough level ± SEM, ng/ml	Target value, ng/ml
TAC	4	11.4 ± 3.6	10–15
SIR	3	11.6 ± 3.8	10–15
MMF	30	3.8 ± 1.2	3.5–5.5

$n = 5$ per group.